Faecalibacterium prausnitzii subspecies–level dysbiosis in the human gut microbiome underlying atopic dermatitis

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Background: Atopic dermatitis (AD) is a serious global epidemic associated with a modern lifestyle.
Objective: Although aberrant interactions between gut microbes and the intestinal immune system have been implicated in this skin disease, the nature of the microbiome dysfunction underlying the disease remains unclear.
Methods: The gut microbiome from 132 subjects, including 90 patients with AD, was analyzed by using 16S rRNA gene and metagenome sequence analyses. Reference genomes from the Human Microbiome Project and the KEGG Orthology database were used for metagenome analyses. Short-chain fatty acids in fecal samples were compared by using gas chromatographic–mass spectrometric analyses.
Results: We show that enrichment of a subspecies of the major gut species *Faecalibacterium prausnitzii* is strongly associated with AD. In addition, the AD microbiome was enriched in genes encoding the use of various nutrients that could be released from damaged gut epithelium, reflecting a bloom of auxotrophic bacteria. Fecal samples from patients with AD showed decreased levels of butyrate and propionate, which have anti-inflammatory effects. This is likely a consequence of an intraspecies compositional change in *F prausnitzii* that reduces the number of high butyrate and propionate producers, including those related to the strain A2-165, a lack of which has been implicated in patients with Crohn disease.
Conclusions: The data suggest that feedback interactions between dysbiosis in *F prausnitzii* and dysregulation of gut epithelial inflammation might underlie the chronic progression of AD by resulting in impairment of the gut epithelial barrier, which ultimately leads to aberrant T_H2-type immune responses to allergens in the skin. (J Allergy Clin Immunol 2016;137:852-60.)

Key words: *Atopic dermatitis, gut microbiota, microbiome, dysbiosis,* Faecalibacterium prausnitzii

Abbreviations used
AD: Atopic dermatitis
CDS: Coding DNA sequence
GalNAc: N-acetylgalactosamine
HMP: Human Microbiome Project
KO: KEGG Orthology
OTU: Operational taxonomic unit
PTS: Phosphotransferase
RFE-SVM: Recursive feature elimination support vector machine
SCFA: Short-chain fatty acid

The chronic inflammatory, noncontagious, and pruritic skin disorder atopic dermatitis (AD) affects up to 25% of children and 2% to 3% of adults in the United States.[1] In general, the incidence of atopic disorders has increased over the last few decades, especially in industrialized countries, suggesting that a modern lifestyle is a major contributing factor to this global epidemic.[1,2] The hygiene hypothesis states that reduced exposure to microbes in early childhood affects the natural development of the immune system or immune tolerance, resulting in an increased susceptibility to allergic diseases.[3,4] Alternatively, the diet-microbiome hypothesis emphasizes that changes in the westernized diet reflecting a lower intake of fiber might lead to changes in the gut microbiome, followed by decreased production of immunomodulatory products, in particular short-chain fatty acids (SCFAs), which have anti-inflammatory effects and contribute to the maintenance of epithelial barrier function.[5,6] The inflamed epithelium with impaired barrier function has been associated with various disorders, including atopic eczema, celiac disease, and Crohn disease.[7-9]

Although the direct cause and pathophysiology of AD are poorly defined, skin damage in patients with AD is likely caused primarily by aberrant T_H2-type immune responses, resulting in overproduction of proinflammatory cytokines against common environmental allergens.[5,10,11] Concurrent dysbiosis in the skin flora has been observed in association with AD, and such changes include an increase in *Staphylococcus aureus* and a decrease in the overall diversity of the microbial community.[12] The results of decades of research suggest that imbalances in certain gut bacterial species are associated with atopic disorders.[13,14] In addition, low microbial diversity in the microbiota in the first month of life has been associated with subsequent incidences of AD.[15] Although these studies have stressed the significance of dysbiosis in the gut microbiota in patients with AD,[14,16] the specific microbial dysfunction that adversely affects the regulation of inflammation underlying AD remains unknown.

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METHODS

Study subjects and fecal sample collection

Ninety patients with AD were recruited for this research among the outpatients of Korea University Anam Hospital in Seoul, Korea, who came from various parts of Seoul, a city of more than 10 million residents, and 42 subjects without AD were chosen from volunteers from the same area (see Table E1 in this article's Online Repository at www.jacionline.org). Patients with AD were given a diagnosis based on the SCORAD scoring system, and the control group (subjects without AD) was defined by a lack of history of visible signs of skin damage indicative of AD. None of the subjects received antibiotic treatment within 6 months before fecal sample collection. The Ethics Committee of the Anam Hospital of Korea University approved this study, and participants or their parents (in cases in which the participants were <17 years old) provided written informed consent for the study. Fecal samples of approximately 5 g were obtained from each subject and stored at −80°C until DNA extraction.

DNA sample preparation

Fecal samples were ground in liquid nitrogen with a mortar and pestle, and 400 mg of each ground sample was transferred to 4 microcentrifuge tubes. Five hundred microliters of a solution containing 0.1-mm-diameter zirconia/silica beads (BioSpec Products, Bartlesville, Okla), 500 μL of extraction buffer (200 mmol/L Tris [pH 8.0], 200 mmol/L NaCl, and 20 mmol/L EDTA), 210 μL of 20% SDS, and 500 μL of phenol/chloroform/isoamyl alcohol mixture (25:24:1, pH 7.9) was added to each tube. The bacterial content in the samples was released by disrupting the cell envelope with a bead beater (BioSpec products) for 2.5 minutes at room temperature. DNA was extracted from the supernatant with phenol/chloroform/isoamyl alcohol (25:24:1, pH 7.9) and precipitated with isopropanol. DNA pellets were dried and dissolved in TE buffer (10 mmol/L Tris [pH 8.0] and 1 mmol/L EDTA), and the concentration was adjusted to 100 ng/μL.

Sequencing of the V1-V2 16S rRNA gene regions

A PCR reaction targeting the V1-V2 region of the 16S rRNA gene was conducted with each DNA sample in a 25-μL reaction mixture containing 100 ng of template DNA, 5 μL of HotStarTaq PCR buffer (Qiagen, Germantown, Md), 1 U of HotStarTaq DNA polymerase (Qiagen), and 0.4 μmol/L each of forward and reverse primer. The forward primer, 5′-CGTATCGCCTCCCTCGCGCCATCAGNNNNNNNN**TC***AGAGTTTGATCCTGGCTCAG*-3′, was a composite of the 454 Titanium adapter A (underlined nucleotides), a unique 8-base barcode (shown with 8 Ns), a linker (the dinucleotide TC), and the universal bacterial primer 8F (denoted in italics). The reverse primer, 5′-CTATGCGCCTTGCCAGCCCGCTCAGNNNNNNNN**CA***TGCTGCCTCCCGTAGGAGT*-3′, was a composite of the 454 Titanium adapter B (underlined), a unique 8-base barcode (Ns), a linker (CA), and the broad-range bacterial primer 338R (in italics). DNA amplification was conducted at 95°C for 15 minutes, followed by 34 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute, with a final extension of 72°C for 5 minutes. PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen). Amplicons were quantified with the PicoGreen Assay (Invitrogen, Life Technologies, Grand Island, NY) and diluted to 1×10^9 molecules/μL in TE buffer. A sequencing library containing equimolar amounts of each product was constructed for 454 FLX Titanium pyrosequencing (Roche, Mannheim, Germany).

Analysis of the 454 sequencing reads

All barcode-tagged sequence reads from the V1-V2 region of the 16S rRNA gene were processed by using the QIIME pipeline.[17] Sequences lacking either the 5′ and 3′ primer sequences or those that contained 1 or more ambiguous bases were removed. All primer sequences were trimmed from the reads, and any trimmed sequences shorter than 250 bp were removed. The sequences were clustered into operational taxonomic units (OTUs) by using CD-HIT[18] based on 97% sequence similarity and were taxonomically identified by using RDP classifier[19] with a minimum confidence score of 0.5. Chimeric sequences were identified and removed by using ChimeraSlayer and BlastFragment in the QIIME pipeline. The Shannon index was calculated to estimate the α diversity of each microbiota, and for β diversity analysis, unweighted UniFrac or Bray-Curtis distance metrics were constructed with QIIME scripts.

Enterotype analysis was conducted by using the partitioning around medoids clustering algorithm[20] with a genus-level distance matrix that was constructed with the Jensen-Shannon divergence algorithm.[21] The Calinski-Harabasz index was calculated to estimate the optimal number of enterotype clusters, which was 2 for our data (Fig 1).

As an alternative approach to OTU construction based on CD-HIT,[18] ESPRIT[22] was used to sort the 454 sequencing reads into taxon-independent OTUs that were grouped at various distance levels (equivalent to identity levels of 90% to 97%). The OTU abundance matrix that was constructed with these OTUs was used to identify the most discriminatory OTUs between the AD and non-AD microbiota by using the recursive feature elimination support vector machine (RFE-SVM).[23] OTUs that were not present in at least 30% of the samples were removed from the data set to reduce the noise level in the OTU data. Leave-one-out cross-validation was used to estimate the prediction accuracy for the AD microbiota from that of control subjects without AD with the selected set of discriminatory OTUs.

Whole-genome shotgun sequencing and sequence analysis

The whole-genome sequences of 8 fecal samples (4 from patients with AD and 4 from healthy subjects) were sequenced by using Illumina HiSeq2000 (Illumina, San Diego, Calif). A DNA library with a fragment length of approximately 400 bp was prepared, and paired-end reads of 101 bp were obtained from both ends. Sequence analysis was conducted with the CLC Genomics Workbench (CLC bio, Aarhus, Denmark). Low-quality reads were trimmed based on the following parameters: all sequences of less than the quality score of 0.05 or with more than 2 ambiguous nucleotides. Sequences shorter than 15 bp were also discarded. Functional profiles of the metagenome were analyzed by using HUMAnN v0.99.[24] All recently released *Bacteroides* and *Faecalibacterium* genomes (bfg, bhl, bsa, bxy, bdo, bdh, bacc, fpr, and fpa) were added to the KEGG Orthology (KO) database v54 for analysis. We conducted a translated BLAST search of the Illumina reads against the KO database by using USEARCH v8.0[25] under the following conditions: (usearch_local command with –id 0.3 –evalue 1.0 –maxaccepts 20 –maxrejects 256). The results were passed through HUMAnN by using default parameters. Then the normalized orthologous gene family abundances were compared between the AD and non-AD groups.

Pangenomic analysis of the 5 *F prausnitzii* strains

The genome sequences of the 5 *F prausnitzii* strains were downloaded from the Human Microbiome Project (HMP) Web site (http://www.hmpdacc.org/) and compared to construct the pangenome. We compared all coding DNA sequences (CDSs) from the strains by using BLASTP and clustered the related CDSs into discrete orthologs based on both amino acid level similarity and matching regions of 70% or greater. All CDSs from the 5 genomes were annotated with the KO database[26] based on the best BLASTP hits, with an e-value cutoff of 1e-5.

The whole-metagenome reads from Illumina HiSeq2000 sequencing were mapped on the genomes of the 5 *F prausnitzii* strains by using the CLC Genomics Workbench (CLC bio) with the following settings: mismatch cost, 2; insertion cost, 3; deletion cost, 3; length fraction, 0.5; similarity fraction, 0.8; auto detect paired distances, yes; global alignment, no; and nonspecific match handling, map randomly. As a result, 10.14% of the Illumina reads were mapped on the *F prausnitzii* genomes with 1.19% SE on average.

Determination of SCFA levels in fecal samples

Fecal samples were homogenized in liquid nitrogen with a mortar and pestle, and approximately 0.1 g of the crushed samples was transferred to 2-mL microfuge tubes. An internal standard (60 μL of 0.25 mmol/L 4-methylvaleric acid) was added to a tube containing a crushed fecal sample

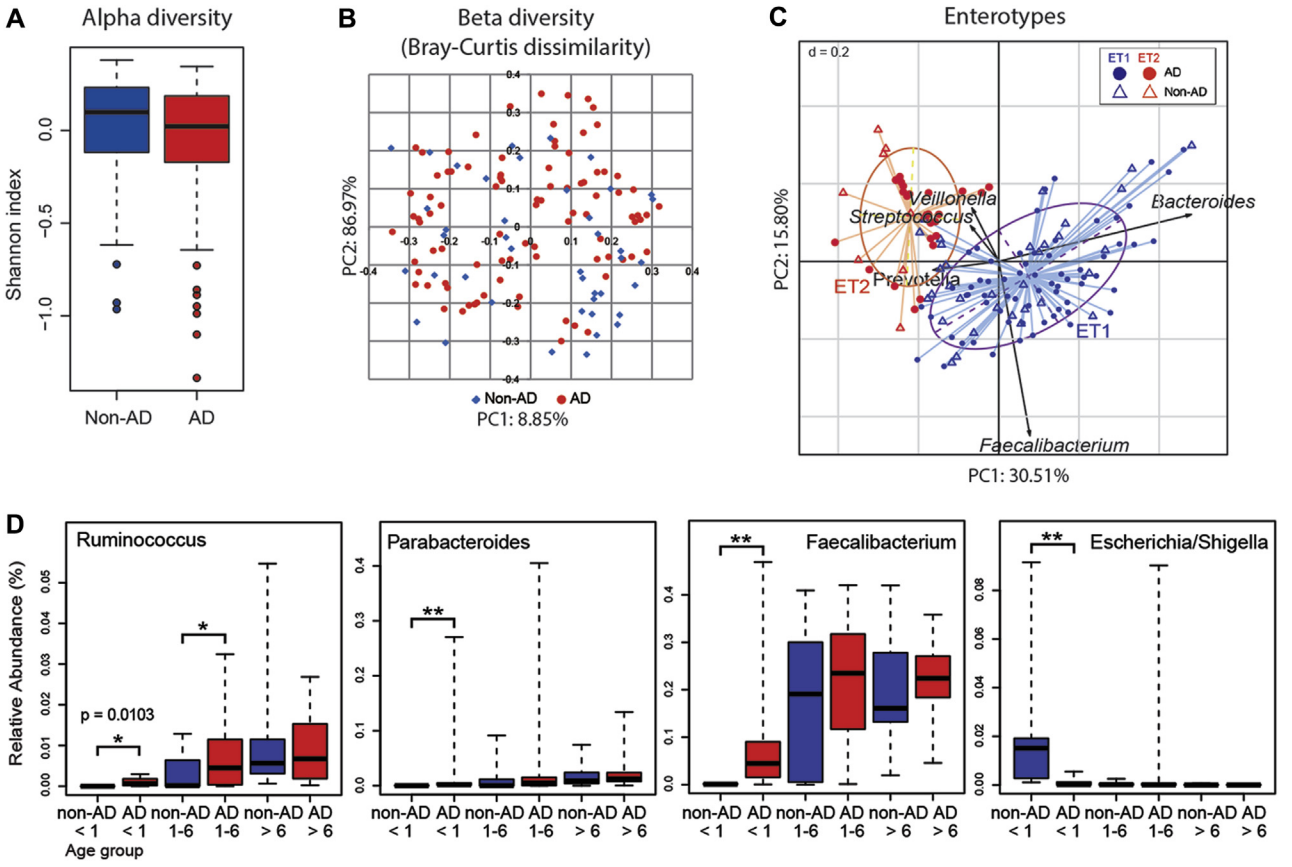

FIG 1. Diversity profiles of the gut microbiota. **A,** Box plots of α diversity (internal diversity) in each microbiota comparing patients with AD and control subjects without AD. **B,** β-Diversity among samples. **C,** Enterotype patterns. **D,** Bacterial genera that are distinctive between the AD and non-AD microbiota. **P* < .05 and ***P* < .01.

to measure SCFA levels in fecal samples. After acidification of the sample with 20 μL of 33% HCl, 1 mL of diethyl ether was added, and the tube was vortexed for 10 minutes. The diethyl ether layer was separated by means of centrifugation and transferred to a new tube. A second diethyl ether extraction procedure was conducted, and the diethyl ether phase from the 2 extractions was combined. Sixty microliters of the extract and 20 μL of *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide were mixed in a glass insert in a gas chromatography autosampler vial and incubated for 2 hours at room temperature. Gas chromatography–mass spectrometry was conducted by using the prepared samples, as previously described.[27] Ratios of D-lactate and L-lactate were measured by using the Megazyme enzymatic kit (Megazyme, Wicklow, Ireland). Frozen fecal samples (0.1 g) were dissolved in 1 mL of 0.1 mol/L triethanolamine buffer (pH 9.15) and centrifuged at 14,000*g* for 10 minutes at 4°C. The supernatant was precipitated with 6.1 N trichloroacetic acid (10% final concentration) and centrifuged at 4500*g* for 20 minutes at 4°C. Precipitated supernatants were used to measure lactate levels, according to the manufacturer's instructions.

Promoter activity analysis

To compare promoter activity of the butyryl CoA:acetate CoA-transferase gene in the 5 sequenced *F prausnitzii* strains (FP2_20620 for strain L2-6, FAEPRAA2165_01575 for strain A2-165, HMPREF9436_00973 for strain KLE1255, FPR_29560 for strain SL3/3, and FAEPRAM212_02812 for strain M21/2), a *lacZY* fusion was made to the synthesized promoter of each gene by using the promoter probe vector pLKC481,[28] and the plasmid constructs were used to transform *Escherichia coli* DH5α.[29] *E coli* strains containing different pLKC481 constructs were grown in LB broth to mid-log phase (OD_{600} = 0.7) and incubated on ice for 20 minutes. Two milliliters of each culture was harvested by means of centrifugation at 4°C for 10 minutes, and the pellets were resuspended in Z buffer.[29] β-Galactosidase activity, representing promoter activity, was assayed with O-nitrophenyl-β-D-galactoside as the substrate and expressed as Miller units calculated as follows:

$$1000 \times (OD_{420} - 1.75 \times OD_{550}) / (\text{Time of incubation [in minutes]} \times \text{Volume [in milliliters]} \times OD_{600}).$$

RESULTS

Diversity profiles of the AD microbiota

DNA representing the gut microbiota from 132 subjects, including 90 patients with AD, was isolated from fecal samples, and the V1-V2 regions in the 16S rRNA gene pool were amplified and sequenced by using 454 technology. The sequence reads were processed to clean low-quality sequences, resulting in an average sequencing depth of 6604 reads per sample. No significant difference was observed in the microbial diversity (α diversity) of all AD microbiota compared with that of all non-AD microbiota (Fig 1, *A*). Likewise, the dissimilarity in composition between the AD and non-AD microbiota (β diversity) was not significant (Fig 1, *B*). Each of the microbiota can be assigned to an enterotype based on the major bacteria groups, although enterotyping might not be as informative as first proposed.[30,31] Nevertheless, the 132 microbiota were best clustered into 2 enterotypes, *Bacteroides* and *Prevotella* types, but the AD microbiota did not

significantly partition into a particular enterotype (Fig 1, *C*). Together, these data show that global-scale changes are not significantly different between AD and non-AD microbiota. However, we observed specific changes in some bacterial clades in subsets of the 90 AD microbiota divided by age (Fig 1, *D*, and see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). Although there have been reports of bacterial clade enrichment belonging to the family Enterobacteriaceae and decreases in those belonging to *Lactobacillus* and *Bifidobacterium* species in patients with AD,[13,14] these patterns were not observed in our data. Differences in the patients with AD (eg, race and diets) might be responsible for the apparent discrepancy between previous reports and this study. In addition, at least some of the bacteria reported as AD linked in previous studies might be the ones to bloom often in the damaged gut environment and not necessarily associated with AD.[32] Bacteria that are significantly associated with the AD microbiota can include those that have a pathogenic role in patients with AD, or they might simply have a growth advantage in the changed gut environment. Pathogenic bacteria that play a pivotal role in patients with AD can be specific, possibly a single species or a consortium of a few bacteria,[32] and their levels can be increased under all chronic AD conditions. By contrast, those that are simply benefited by the disease might involve diverse bacteria. As a whole, their levels might be increased in all AD microbiota; however, individually, they might not be present in all AD conditions.[32] Considering these patterns, most bacterial clades increased in the AD microbiota (Fig 1 and see Fig E1) appear to be species simply taking advantage of growth conditions in the guts of patients with AD. However, it is possible that even if there were specific bacteria associated with all AD microbiota, their small numbers were difficult to tease out because of the overwhelming presence of other species present within the same clade.

We conducted a RFE-SVM analysis[33] to select for the top discriminatory OTUs that are distinctive between AD and non-AD microbiota. For this analysis, we used the OTUs constructed by using ESPRIT, which is based on taxonomy-independent hierarchical classification (at the 0.03 distance level, which generally matches the species level).[22] Half of the random selection from the data was used as a training set for the RFE-SVM analysis to select for discriminatory OTUs. The other half of the data were used to test these OTUs for differentiating AD status. The OTUs showed an accuracy of 88.14% on average, whereas the control run with random OTUs showed an accuracy of 79.92% on average (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). We found that many of the top discriminatory OTUs belong to a few bacterial clades, some of which are common to all ages (see Fig E2, *B*). *Faecalibacterium* species is of particular interest because it was also shown to be significantly increased in AD microbiota (Fig 1) and represents only a single known species, *F prausnitzii*, although this species can be divided into different phylogroups with possible physiologic differences.[34]

AD-specific subspecies of *F prausnitzii* OTUs

Because *F prausnitzii* was distinctly altered in the AD microbiota (Fig 1, *A*), was one of the most significant species in the RFE-SVM model (see Fig E2), and is one of the most abundant species in the human gut microbiota, suggesting its crucial role in general human physiology,[35] the evidence suggests the possibility that *F prausnitzii* plays a pivotal role in patients with AD. Furthermore, because *F prausnitzii* is a single species, it is easier to study the composition of the clade. To explore the possible presence of subspecies more associated with AD than the species as a whole, we explored *F prausnitzii* OTUs. The results show that the ESPRIT OTUs annotated as *F prausnitzii* were clustered into 7 distinct clades in the phylogenetic tree (Fig 2, *A*). This tree is based on V1-V2 sequence identity, and therefore there is a possibility that each clade can be further divided when the entire sequence of the 16S rRNA gene is used. Nevertheless, we found that one of the clades, F06, was significantly enriched in the AD microbiota (Fig 2, *A* and *B*). Enrichment of the F06 OTUs in the AD microbiota was common

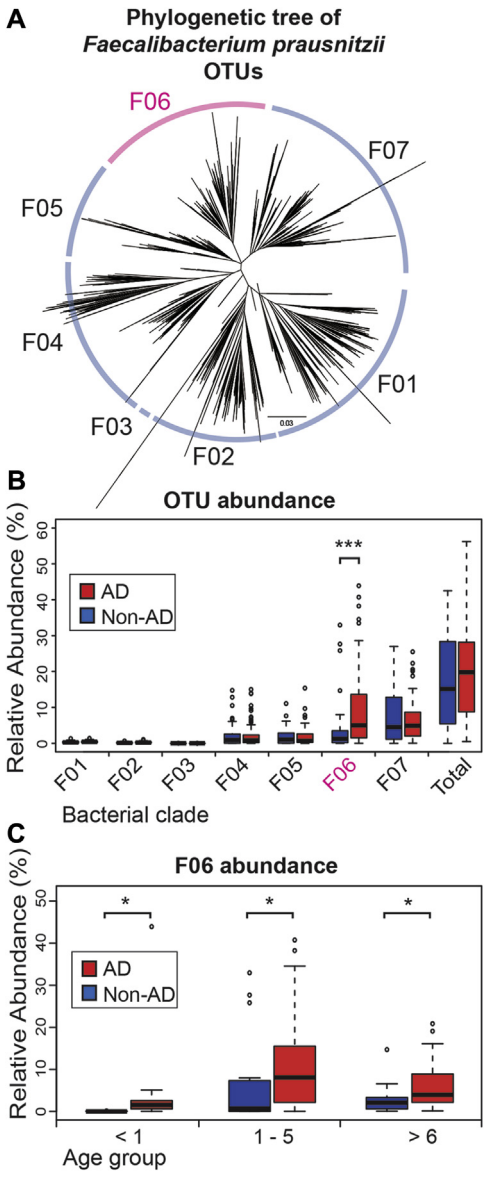

FIG 2. The phylogenetic tree of *F prausnitzii* OTUs. **A,** The *F prausnitzii* tree consists of 7 clades based on the V1-V2 sequence of the 16S rRNA gene. **B,** The OTUs belonging to F06 are significantly more enriched in the AD microbiota than in the non-AD microbiota. **C,** Enrichment of the F06 OTUs in the AD microbiota is observed across all age groups. *Asterisks* indicate significant differences: $*P < .05$ and $***P < .003$.

to all ages but was most distinct in the youngest group (<1 year old; Fig 2, *C*). This is intriguing because AD often occurs within the first year of life,[1] and this enrichment pattern suggests the possibility that *F prausnitzii* subspecies might play a role in the onset of the disease.

Five strains of *F prausnitzii*, A2-165, M21/2, L2-6, S3L/3, and KLE1255, have been sequenced through the HMP.[36] The 16S rRNA gene sequence of one of these strains, L2-6, has a unique signature in the V1-V2 region, particularly in the V1 region, which is distinctive from that of the other strains but closely matches the F06 clade (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). Although the V1-V2 region is only one part of the 16S rRNA gene, the almost perfect match over this entire region (see Fig E3, *B-D*) suggests the close kinship between L2-6 and F06 bacteria.

By conducting a cross-comparison of the predicted proteins from all 15,825 genes in the 5 genomes with BLASTP (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov), we determined the *F prausnitzii* pangenome was comprised of 7,253 orthologous genes (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). The strain L2-6 had 855 unique genes, and the other strains had a similar number of unique genes. A subset of 8 fecal samples (4 each of AD and non-AD samples) were subjected to whole-genome shotgun sequencing by using the Illumina HiSeq 2000 platform to compare the gut microbiome contents in relation to the *F prausnitzii* pangenome, generating sequence reads at an average depth of 552 Mb per sample. These 8 fecal samples were chosen based on similar and high *F prausnitzii* content (19% to 36%; see Table E2 in this article's Online Repository at www.jacionline.org). When the Illumina reads were mapped to the *F prausnitzii* pangenome, most (87.4%) of the genes unique to L2-6 had significantly higher matches to the reads from the AD metagenome than those from the subjects without AD (see Fig E4, *B* and Table E3 in this article's Online Repository at www.jacionline.org). In contrast, the unique genes of A2-165 and KLE1255 had significantly more matched reads from the non-AD metagenome (see Fig E4, *B*). Together, these data suggest that organisms belonging to clade F06 and the strain L2-6 are not only related at the V1-V2 region of the 16S rRNA gene but also at the genomic level, further supporting their close kinship. Such a connection between phylogenetic information and genomic content in bacteria is due to shared evolutionary history, which has previously been observed and discussed.[37]

Metabolic functions affected in the AD microbiome

We explored metabolic dysfunction of the AD microbiome in 2 ways, first by mapping the Illumina reads to the *F prausnitzii* pangenome as the reference and second by mapping the same Illumina reads to the KO database (see the Methods section). The *F prausnitzii* pangenome approach of metagenomic analysis is effective because there is high *F prausnitzii* content (19% to 36%) in the metagenome (see Table E2). Although this pangenome is based on only 5 strains, it includes 2 biologically significant strains: A2-165, a lack of which is associated with Crohn disease,[28] and L2-6, levels of which were significantly increased in the AD microbiome, as shown in this study (see Figs E3 and E4). To interpret the mapped data in terms of metabolic function, the *F prausnitzii* pangenome was annotated by using the KO database, and the KOs with significantly ($P < .05$) different numbers of matching Illumina reads between the AD and non-AD microbiomes were collected. A total of 1332 KOs were assigned to the pangenome, and 415 of these showed significant imbalances; 122 KOs were enriched in the AD microbiome, and 293 KOs were enriched in the non-AD microbiome (Fig 3, *A*, and see Table E4 in this article's Online Repository at www.jacionline.org).

The KOs that were significantly increased in the AD microbiome included those encoding metabolic functions and pathways responsive to oxidative stress (peroxiredoxin, dimethyl sulfoxide reductase, and *sufBC* operon),[38-40] various transition metal transporters (Fe, Ni, Zn, and Mn) that might counteract nutrient metal restriction exerted by the host,[41] and those involved in use of a major mucin component, N-acetylgalactosamine (GalNAc; a phosphotransferase (PTS) system for uptake and catabolic enzymes; Fig 3, *B*).[42] In contrast, genes with decreased content in the AD microbiome included those involved in the biosynthesis of various amino acids, nucleotides, peptidoglycans, and cofactors (cobalamin, coenzyme A, and thiamine; Fig 3, *A*). Together, the gene abundance profiles in the AD microbiome might reflect the gut environment, with tissue damage resulting from increased inflammation in the gut epithelium. The damaged gut environment might have increased levels of host-derived general nutrients, such as amino acids, cofactors, and coenzymes, as well as specific nutrients, including the mucin component GalNAc,[42] the use of which requires specific machinery for uptake and catabolism (Fig 3, *B*). The increased supply of additional nutrients might result in the microbiome-wide loss of basic biosynthetic gene content, whereas the presence of specific nutrients, such as GalNAc, can apply selective pressure for auxotrophic specialists or pathobionts that are capable of using them. Such a gut environment might in turn lead to further gut epithelial tissue breakdown, completing the circle to cause dysbiosis of the gut microbiota and thereby microbial dysfunction. It is intriguing to note that among the 5 *F prausnitzii* reference strains, only L2-6 contains the gene cluster for GalNAc use (Fig 3, *B*). In *E coli* O157:H7 a PTS system for GalNAc uptake was shown to be required for the bacterium to colonize the bovine gut epithelium.[43]

From the second analysis using the KO database to map the Illumina reads, 122 of 5832 KOs showed significant differences between the AD and non-AD metagenomes (see Fig E5 and Table E5 in this article's Online Repository at www.jacionline.org). Levels of a total of 66 and 56 KOs were significantly increased in the AD and non-AD metagenome, respectively. Although the resolution of the KO analysis was lower than that of the *F prausnitzii* pangenomic analysis, the KO analysis provides value because it includes more metagenome-level activity beyond that of just the *F prausnitzii* component. Consistent with the *F prausnitzii* pangenomic analysis, the KO analysis showed the same pattern of increases in metal transporters, GalNAc use, and oxidative stress modulation and decreases in the biosynthesis of amino acids, nucleotides, and cofactors in the AD metagenome (see Fig E5). However, there were new genes that were found to be increased in the AD metagenome, such as those encoding L-fucose use (L-fuculokinase and L-fucose isomerase). L-fucose is released from mucin glycoproteins and serves as a major nutrient component in mucin other than GalNAc for many gut bacteria, including *Bacteroides thetaiotaomicron*.[44] More new genes include those encoding the glutathione S-transferase family, glucose-6-phosphate 1-dehydrogenase, glutamate decarboxylase, and glyoxylate and dicarboxylate metabolism (see Fig E5). Glutathione is a tripeptide of cysteine and glutamate,

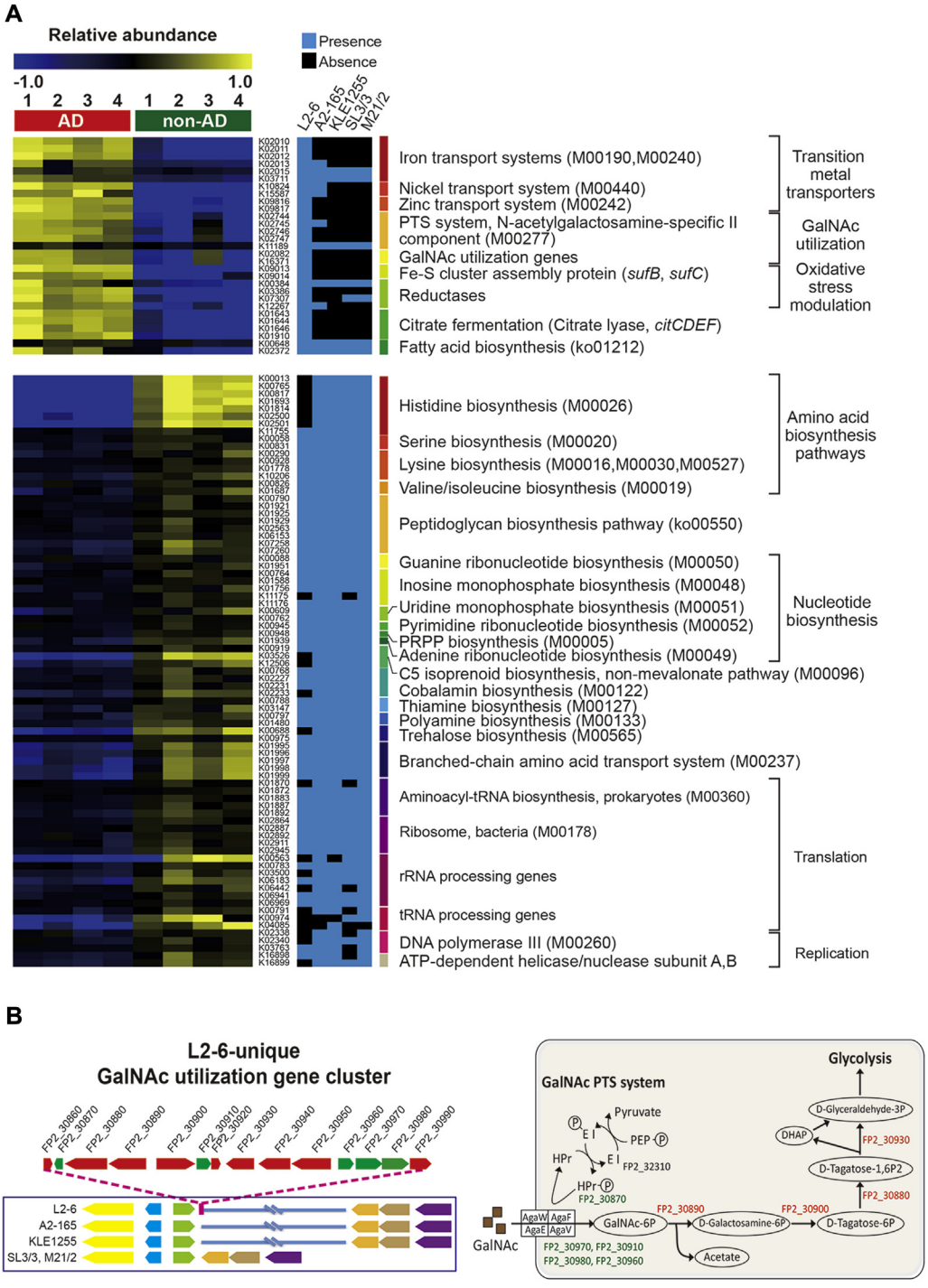

FIG 3. *F prausnitzii* pangenomic genes that are significantly altered in the AD microbiome. **A,** Heat map of significantly altered functions in the AD microbiome. KOs from the *F prausnitzii* pangenome that exhibit a shift in abundance profile between the AD and non-AD microbiomes are shown. Relative abundance of the matching Illumina sequence reads to a given KO is displayed as a $\log_2$ ratio. For some groups of KOs, module *(M)* or pathway *(ko)* numbers are also shown. The presence of KOs in each *F prausnitzii* strain is also shown. **B,** The gene cluster encoding GalNAc use that is uniquely present in the L2-6 strain among the *F prausnitzii* strains. The GalNAc PTS system and the catabolic pathway encoded by the gene cluster are also shown.

which allows bacteria to maintain homeostasis during oxidative stress caused by highly reactive oxygen and nitrogen metabolites produced during inflammatory cascades.[45] Glutathione-S-transferases catalyze conjugation of the reduced form of glutathione to endogenous compounds, such as peroxidized lipids, for the purpose of detoxification. Glucose-6-phosphate 1-dehydrogenase levels have been shown to be increased in *E coli* under oxidative stress, leading to increases in antioxidant

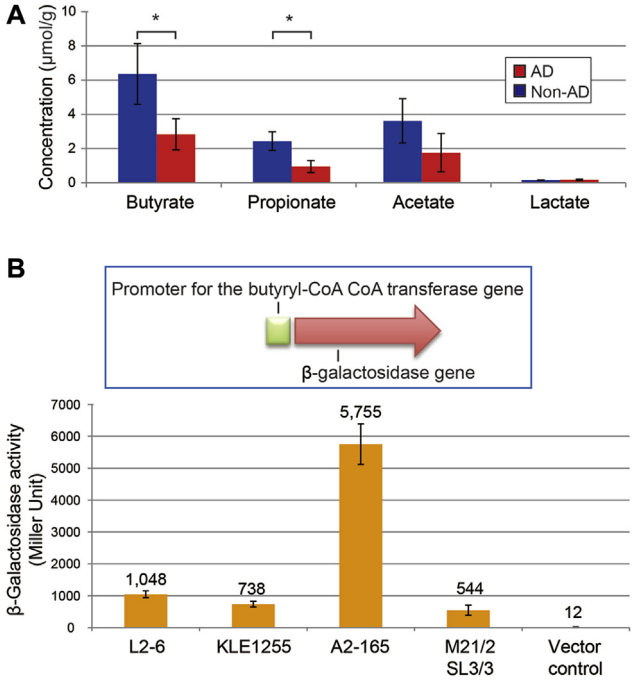

FIG 4. Analyses of SCFAs in fecal samples. **A,** Gas chromatographic–mass spectrometric quantification of selected SCFAs in AD and non-AD fecal samples. *$P < .05$. **B,** Comparison of butyryl CoA:acetate CoA-transferase gene promoter activity from *F prausnitzii* strains.

molecules glutathione and nicotinamide adenine dinucleotide phosphate.[46] Glutamate decarboxylase has been shown to play an important role in antioxidation in *E coli* O157:H7 and *Saccharomyces cerevisiae*.[47,48] In addition, glyoxylate and dicarboxylate metabolism was shown to be involved in the antioxidation process.[49] Together, this abundance pattern of the genes augments the notion that the AD gut epithelium has tissue damage resulting from increased inflammation, as suggested by the *F prausnitzii* pangenomic analysis (Fig 3).

Because levels of a variety of auxotrophs or pathobionts were expected to be increased, the distribution of the gene family coding for the GalNAc-specific IIA component in the PTS system (*agaF*, K02744), one of the key genes in the cluster, was examined in the AD microbiome, and extensive diversity was observed (see Fig E6 in this article's Online Repository at www.jacionline.org). Cross-species enrichment of the genetic capacity for mucin component use, although the actual activity or function of each gene remains to be verified, suggests that the AD gut environment is a favorable milieu for the establishment of a variety of auxotrophs and pathobionts other than the L2-6 strain, which might have implications for the exacerbation and chronic nature of AD.

F prausnitzii F06 subspecies blooms might lead to a reduction in butyrate and propionate production

Production of SCFAs by the gut microbiota has tremendous health benefits.[50,51] To compare the production of these metabolites between the AD and non-AD microbiome, we conducted gas chromatographic–mass spectrometric analyses on fecal samples (Fig 4, *A*). Levels of major SCFAs, acetate, propionate, butyrate, and D- and L-lactate were measured and compared between paired AD and non-AD fecal samples that were selected for similarly in high total *F prausnitzii* levels but also with significant differences in F06 OTU levels (Fig 4, *A*). Because the total level of *F prausnitzii* in these samples was much higher than levels of other SCFA producers, including *Eubacterium rectale*, *Eubacterium hallii*, and *Roseburia* species (see Table E6 in this article's Online Repository at www.jacionline.org), the assay results could be directly linked to changes in *F prausnitzii* composition. Comparative analysis of the 12 pairs of samples showed that the low F06 microbiome samples had more butyrate and propionate than the paired high F06 microbiome samples (Fig 4, *A*). Such a major change in SCFA concentration and ratio in the patients with AD compared with the subjects without AD is significant because it suggests major differences in the gut environment between the 2 groups. Additional differences between the AD and non-AD groups, including age in some pairs, did not seriously affect the SCFA profiles, suggesting that the F06 level is the most important contributing factor determining the SCFA profile. It is noteworthy that propionate was recently shown to have an antiallergic effect; it impaired the capacity of dendritic cells to provoke T_H2 cell–mediated allergic airway inflammation.[51] On the other hand, there were no significant differences in acetate levels, total lactate levels, or the ratio between L- and D-lactate levels, which was previously shown to be associated with disorders (Fig 4, *A*).[50] Although changes in SCFAs are a likely mechanism for the proposed effects of *F prausnitzii* subspecies, additional anti-inflammatory agents that can be produced by *F prausnitzii* also remain to be explored.

Because the L2-6 strain, which is related to the F06 clade, and the other 4 strains have the same butyrate biosynthesis pathway (see Fig E7 in this article's Online Repository at www.jacionline.org), we investigated whether a difference in the expression of the pathway, particularly the gene coding for the key enzyme butyryl CoA:acetate CoA-transferase, could be detected. The promoter region of this gene from each strain was synthesized and cloned into a promoter-probe vector[28] upstream of the *lacZ* reporter gene, and gene expression was compared in *E coli* (Fig 4, *B*). Although the assay was not conducted in the natural *F prausnitzii* host, the basic strength of the promoters could be compared among strains because any regulatory function that is not properly operational in *E coli* would be equally applied to the promoters. The results show that the promoter from the A2-165 strain is significantly stronger than the promoters from L2-6 and other strains (Fig 4, *B*). In fact, these data are consistent with a concordant result that showed differential butyrate production in the A2-165 and L2-6 strains,[52] although the same pattern was not observed when a different growth medium was used.[53] Together, these data suggest that different butyrate levels in fecal samples might be largely attributable to different *F prausnitzii* subspecies composition. We conclude that dysbiosis in the *F prausnitzii* species in association with AD might result in suppression of high butyrate producer subspecies, such as the A2-165 type bacteria, which might lead to a reduction in overall butyrate production.

DISCUSSION

A dominant human intestinal bacterial species, *F prausnitzii*, has been considered beneficial to gut health through its production of SCFAs, in particular butyrate, which has anti-inflammatory effects and also serves as a direct source of energy for the gut epithelium.[35,54] Decreased overall levels of *F prausnitzii* and accompanied reduction in SCFA production have been

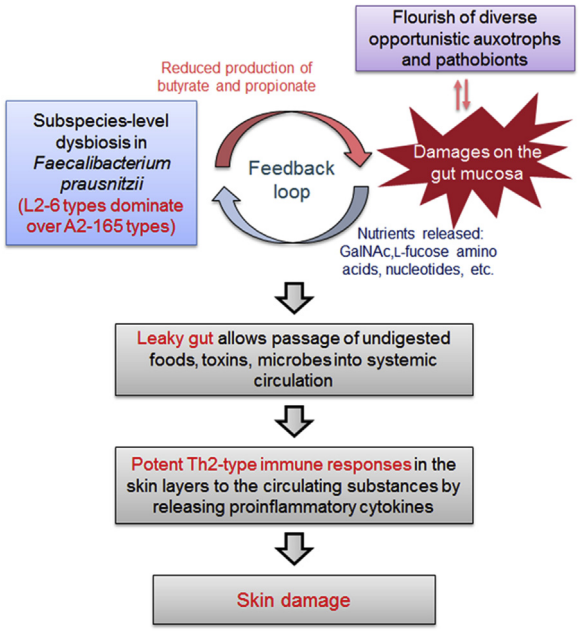

FIG 5. Proposed model of AD in association with dysfunction of *F prausnitzii*. Damaged intestinal mucosa at sites of increased inflammation can release a variety of nutrients, including the core mucin component GalNAc, which stimulates the growth of L2-6–type bacteria and other auxotrophic opportunists. The bloom of poor butyrate producers, such as L2-6–type bacteria, in the *F prausnitzii* population might lead to a decrease in the number of high butyrate producers, such as A2-165–type bacteria. This intraspecies compositional change results in decreased production of butyrate and propionate, which in turn leads to further dysregulation of gut epithelial inflammation, establishing feedback interactions. It is this feedback loop that underlies the onset and chronic progression of AD by maintaining increased permeability in the gut epithelium, which can lead to aberrant T_H2-type immune responses in the skin.

implicated in patients with Crohn disease.[35,54] From the current study, it is evident that the balance among the subspecies of *F prausnitzii*, specifically that between the L2-6– and A2-165–type bacteria, can also result in changes in SCFA production (Fig 5). This study also demonstrates the importance of fine-level characterization of bacterial species to understand the function of the microbiome, which is affected by interactions among closely related bacteria that might compete for the same niche but have distinct activities.

The AD metagenomic data revealed increased numbers of genes encoding use of the major mucin components GalNAc and L-fucose and various nutrients that would be released generally from damaged gut epithelial cells (Figs 3 and 5), which might reflect the increased inflammation in the gut epithelium and correspond in a broad sense to "leaky gut syndrome."[7] When such epithelial damage occurs, possibly triggered by dysbiosis in *F prausnitzii* or by some other unknown causes, this might in turn lead to dysbiosis in *F prausnitzii*, resulting in a bloom of various pathobionts or auxotrophs and establishing a feedback loop between dysbiosis in *F prausnitzii* and dysregulation of gut epithelial inflammation. Epithelial damage has been associated with atopic eczema and many diseases, including celiac disease, alcoholism, Crohn disease, chronic giardiasis, and intestinal candidosis.[7] The damaged epithelium with increased permeability might allow for the passage of undigested foods, toxins, and pathogenic microbes into the systemic circulation, ultimately allowing harmful substances to reach the skin (Fig 5).[9,55] Although much of the humoral process needs to be explored, studies suggest that the aberrant T_H2-type immune responses might react to these foreign substances by releasing proinflammatory cytokines, thus increasing inflammation in the deep layers of the skin and causing damage (Fig 5).[5,56] Numerous studies reporting the positive effects of probiotics and prebiotics on the digestive tract in reinforcing a healthy immune system support the pivotal role played by the gut microbiota in the onset and progression of many diseases.[57,58] Our data suggest that the development of methods to target *F prausnitzii*, the dysfunction of which underlies chronic AD, might hold the key to effective diagnosis of and therapy for AD.

Clinical implications: Our results provide the insights into the basis of AD originated in the gut microbiome, which will lead to the development of effective diagnosis of and therapy for the disease.

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